Review

Thioredoxin targets in plants: The first 30 years

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ABSTRACT

The turn of the century welcomed major developments in redox biology. In plants, proteomics made possible the identification of proteins linked to thioredoxin (Trx), initially in chloroplasts and then other cell compartments. Two procedures, one based on thiol specific probes and the other on mutant Trx proteins, facilitated the labeling or isolation of potential Trx targets that were later identified with proteomic approaches. As a result, the number of targets in land plants increased 10-fold from fewer than 40 to more than 400. Additional targets have been identified in green algae and cyanobacteria, making a grand total of 500 in oxygenic photosynthetic organisms. Collectively these proteins have the potential to influence virtually every major process of the cell. A number of laboratories currently seek to confirm newly identified Trx targets by biochemical and genetic approaches. Almost certainly many new targets become redox active during oxidative stress, enabling the plant to cope with changing environments. Under these conditions, certain targets may be glutathionylated or nitrosylated such that reversion to the original reduced state is facilitated not only by Trx, but also, in some cases preferably, by glutaredoxin. When judging changes linked to Trx, it is prudent to recognize that effects transcend classical light/dark or oxidative regulation and fall in other arenas, in some cases yet to be defined. While future work will continue to give insight into functional details, it is clear that Trx plays a fundamental role in regulating diverse processes of the living cell.

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1.   Introduction

Numerous excellent reviews have been written to describe the roles that different types of Trx play in diverse processes taking place in land plants and oxygen-evolving microorganisms (cited in [1]). Most discuss Trx-linked proteins that have been identified since the first evidence for redox regulation were obtained more than 40 years ago [2]. Moreover, several reviews published in the past few years highlight the striking increase in established and potential targets identified with the development of proteomic-based approaches. However, none of the reviews provides an up-to-date, comprehensive list of established and potential target proteins currently known to be linked to Trx. In this review, we attempt to fill this gap.

2.   Redox regulation of protein function by thiol disulfide exchange

The unique redox, catalytic and metal-binding properties of sulfur are in keeping with its central role in biology. Sulfur can occur in ten different oxidation states *in vivo*, ranging from -2 in thiols (R-SH) to $+6$ in sulfate anion (SO_4^{2-}). Sulfur is present in two amino acids, Met and Cys. In proteins, Met is found primarily in two forms: a principal form in which the sulfur is unmodified and an oxidized form (methionine sulfoxide). By contrast, because of its facility for oxidation, the thiol of Cys can be modified through a range of redox potentials to form a disulfide (S–S) or one of several acids, including sulfenic (R-SOH), sulfinic (R-SO$_2$H) and sulfonic (R-SO$_3$H) acids [3]. This capability enables Cys, which represents a minor fraction of amino acids in proteins (2% in the model plant, *Arabidospis thaliana* [4]), to meet a spectrum of needs of the cell. Disulfide bonds form in many proteins when Cys residues are in sufficiently close proximity. In other instances, a disulfide bond can form with glutathione (GSH) in which case the protein is said to be glutathionylated. Exposed Cys residues can also react with oxygen to be oxidized beyond the disulfide state or with NO and be nitrosylated [3].

Under optimal physiological conditions, the extracellular environment is oxidative while the cytoplasm is reductive. Although the redox potential of the cytoplasm is generally within a range that favors reduction of sulfhydryls, Cys residues are particularly susceptible to oxidation by reaction with reactive oxygen species (ROS) produced at low levels under these conditions. Under optimal or mild oxidative conditions, Cys residues may be oxidized to stable disulfides or less stable sulfenic acids. Intramolecular disulfide bonds may be formed as well as intermolecular or mixed bonds with another protein or with glutathione. Oxidation to the disulfide or sulfenic acid is reversible and native conformations can be readily restored following reduction back to the SH state. Because disulfide bonds are relatively stable, this oxidation state protects the Cys residues from further oxidation. Under highly oxidative conditions, thiol groups can be converted beyond the sulfenic to the sulfinic or sulfonic acid state. Alternatively, they can react with products of lipid peroxidation. These modifications, however, are irreversible and, as a consequence, altered proteins are degraded.

Change in the oxidation state of Cys residues usually leads to an alteration in the structure of proteins that is accompanied by a loss or gain of function, an increase in susceptibility to proteases or denaturation. Cys residues are thus critical to the conformation and stability of proteins and are often essential to protein function [5]. Certain proteins capable of reversible disulfide bridge formation undergo redox-linked changes in activity or related properties. These changes enable biological processes to respond readily to change in redox environment and allow organisms to adapt.

Originally discovered in studies on photosynthesis, regulation of protein function by change in Cys redox state now appears to occur throughout biology. Established redox-regulated processes in plants include carbon assimilation, seed germination, transcription, translation, cell division, redox signaling, radical scavenging and detoxification. Regulation is typically based on the reversible reduction of intramolecular disulfide bridges or thiol disulfide exchanges. In some cases, intermolecular or mixed disulfide bonds are formed transiently with glutathione. Alternatively, a sulfhydryl group can be oxidized to a sulfenate or nitrosylated. While these mechanisms are of interest and are gaining prominence in plants [3,6–9], they are beyond the scope of the present review. Here we focus primarily on changes accompanying the redox transition between the sulfhydryl and disulfide states.

The major thiol-based systems of regulation are linked to thioredoxin (Trx) or glutaredoxin (Grx) [10]. Trx and Grx are small proteins with disulfide reductase activity. Although sharing no sequence similarity, Trx and Grx have similar folding [11] and partly overlapping functions. In addition to their role in redox regulation, Trx and Grx can act as substrates for reductive enzymes such as peroxidases and ribonucleotide reductase—the enzyme that led to the discovery of Trx [12].

Finally, independently of its reductase activity, reduced Trx acts as an essential structural component for phage T7 DNA polymerase [13], in phage assembly [14] and prevention of apoptosis [15]. In this latter capacity, Trx can rather be considered as a redox sensor involved in cell signaling.

Trx-linked functions have been extensively documented for several organisms—initially for *Escherichia coli* and later for yeast and humans (which contain two to three Trx isoforms), and plants—where up to 22 genes have been identified in the genome of *Arabidopsis* [16,17]. Six different types of plant Trx have been described for *Arabidopsis*: *f*, *m*, *x* and *y* in chloroplasts, *o* in mitochondria and *h* in several cell compartments (cytosol, mitochondria, endoplasmic reticulum) as well as outside the cell [18–23]. A similar complexity was found in the genomes of rice [24] and the green alga, *Chlamydomonas reinhardtii* [25]. Current evidence has led to the classification of these six types of Trx in dicots, monocots and green algae. However, exceptions exist. An overview of the Trx isoforms present in the legume, *Medicago truncatula*, recently revealed a novel type in addition to those mentioned above for other plants. This Trx is housed in the endoplasmic reticulum and appears to be dedicated to symbiosis [26]. This new type, present in two isoforms, was designated, "Trx *s*," "*s*" for symbiosis. Thus, a particular type of Trx can be specific to certain plants.

Trxs are members of two redox systems that are found in different cell compartments (Fig. 1). One, the ferredoxin–Trx system (FTS) of oxygenic photosynthesis located in chloroplasts, is comprised of ferredoxin (Fdx), ferredoxin–thioredoxin reductase (FTR) and Trxs *f*, *m* [1], *x* [27] and *y* [28]. In this system, the electrons flow through thiol-disulfide exchange intermediates from Fdx, reduced in the light, to the target protein according to the sequence: Light→Fdx→FTR→Trx→Target Protein. The system becomes oxidized in the dark via molecular oxygen (or oxidized Trx, GSSG or ROS). The chloroplast thus differs fundamentally from the cytoplasm in undergoing change from a reductive state in the light to a more oxidative one in the dark.

The NADP–Trx system (NTS) is localized both in the cytosol and mitochondria [10,29,30]. The system is comprised of NADP–Trx reductase (NTR) that in Arabidopsis occurs in two essentially identical forms, A and B, coded by two distinct genes. The two enzymes have been localized in either the cytoplasm or mitochondria [30]. The enzymes transfer electrons from NADPH to Trx *h* (in the cytoplasm) or Trxs *h* and *o* in mitochondria, according to the sequence: NADPH→NTR→Trx→Target Protein. In addition to the NTS in the cytoplasm and mitochondria, chloroplasts contain a modified type of NTR, designated NTRC, which harbors a Trx domain in a C-terminal extension [31]. This form of the enzyme, which is present only in oxygenic photosynthetic organisms, was recently found to act as a complete NTS in being able to transfer reducing equivalents from NADPH to BAS1—a plastid 2-Cys peroxiredoxin (2-Cys Prx) [32,33]. The redox system functional in the reduction of Trxs *s* in *M. truncatula*, is not known [26].

In the first 25 years that followed the discovery of the FTS in chloroplasts, in 1977, target proteins were identified with biochemical approaches [34]. Although it was likely that unknown targets remained, there was no known way to find them. With the advent of proteomics, procedures were devised to identify unknown target proteins systematically. Developed in 2001, these approaches have been highly successful. Moreover, refinements continue to evolve. In the sections below, we summarize initially Trx targets found in the first 25 years following the discovery of the FTS, and then turn to those found after 2001 with proteomic-based approaches.

3. Thioredoxin targets identified prior to proteomic approaches

Fructose bisphosphatase (FBPase) was the enzyme of chloroplasts that led to the discovery of redox regulation of protein function 40 years ago. Ten years later, in 1977, the members of the chloroplast Trx redox system were identified. Soon thereafter, three other enzymes of the Calvin–Benson cycle—namely phosphoribulokinase (PRK), sedoheptulose bisphosphatase (SBPase) and NADP-glyceraldehyde 3-phosphate dehydrogenase (GAPDH)—were also shown to be linked to Trx [1,35–38]. During this formative period, Trx was found to activate NADP-malate dehydrogenase (NADP-MDH), an enzyme later shown to function in the malate shuttle [39–42]. Trx was also linked to the activation of ATP synthase [43,44] and then to the deactivation
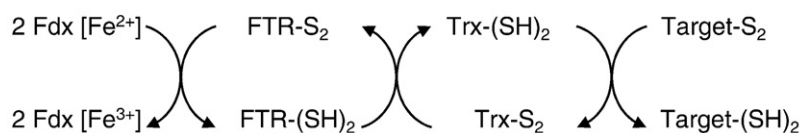
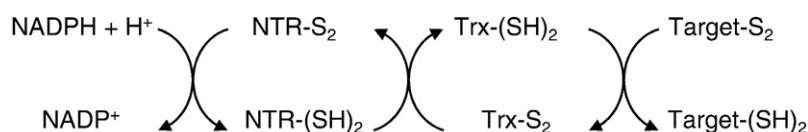Fig. 1 – Ferredoxin–thioredoxin and NADP–thioredoxin redox systems.

Species	Method(s)	Number of identified proteins	Authors
Table 1 – Comprehensive survey of Trx targets in plants and oxygenic photosynthetic microorganisms.			
Organ or organelle			
Land plants			
Arabidopsis			
Etiolated seedlings	Mutant Trxs *h*/affinity trapping SDS-PAGE IEF/SDS 2DE	15	Yamazaki et al., 2004 [86]
Leaves	Trx *h*/labeling (^{14}C-IAM) IEF/SDS 2DE	44	Marchand et al., 2004 [74]
	Trx *h*/labeling (^{14}C-IAM and PEO-biotin) Mutant Trx *h*/affinity trapping IEF/SDS 2DE	71	Marchand et al., 2006 [105]
Thylakoid lumen	Mutant HCF164/affinity trapping SDS-PAGE	9	Motohashi and Hisabori, 2006 [98]
Barley			
Germinating seeds	Trx *h*/labeling (mBBr) IEF/SDS 2DE	3	Marx et al., 2003 [70]
Dry and germinating seeds	Trxs *h*/labeling (mBBr and cyanine 5 maleimide) IEF/SDS 2DE	20	Maeda et al., 2004 [73]
Germinated seed embryos	Trx *h*/labelling (IAM based-ICAT reagents) ICAT based strategy	90	Hägglund et al., 2008 [75]
Chloroplast envelope and stroma	Native and mutant Trxs *f* and *m*/affinity trapping SDS-PAGE	7	Bartsch et al., 2008 [108]
Medicago truncatula			
Dry and germinating seeds	Trx *h*/labeling (mBBr) Mutant Trx *h*/affinity trapping IEF/SDS 2DE	111	Alkhalfioui et al., 2007 [106]
Nicotiana alata			
Styles	Trx *h*/labeling (mBBr) Non-reducing/reducing SDS 2DE Native Trx *h*/affinity trapping SDS-PAGE	1	Juarez-Diaz et al., 2006 [23]
Peanut			
Dry seeds	Trx *h*/labeling (mBBr) Non-reducing/reducing SDS 2DE IEF/SDS 2DE	5	Yano et al., 2001b [69]
Potato			
Chloroplast stroma	Mutant CDSP32/affinity trapping SDS-PAGE	1 6	Broin et al., 2002 [95] Rey et al., 2005 [96]
Rice			
Bran proteins	*E. coli* Trx/labeling (mBBr) IEF/SDS 2DE	4	Yano and Kuroda, 2006 [112]
Spinach			
Chloroplast stroma	Mutant Trxs *f* and *m*/affinity trapping SDS-PAGE	9	Motohashi et al., 2001 [82]
	Mutant Trxs *f* and *m*/affinity trapping IEF/SDS 2DE	37	Balmer et al., 2003 [85]
	Native Trx *f*/affinity trapping IEF/SDS 2DE	28	Balmer et al., 2004b [87]
Thylakoid membrane	*E. coli* Trx/labeling (mBBr) IEF/SDS 2DE	14	Balmer et al., 2006c [107]
Spinach, pea, and potato			
Leaf and tuber mitochondria	Mutant Trxs *m* and *h*/affinity trapping IEF/SDS 2DE	50	Balmer et al., 2004a [88]

Table 1 (*continued*)

Species Organ or organelle	Method(s)	Number of identified proteins	Authors
Wheat			
Mature seed endosperm	*E. coli* Trx/labeling (mBBr) IEF/SDS 2DE	23	Wong et al., 2003 [72]
Developing seed endosperm	*E. coli* Trx/labeling (mBBr) Mutant Trx h/affinity trapping IEF/SDS 2DE	68	Wong et al., 2004 [100]
Amyloplast	*E. coli* Trx/labeling (mBBr) Mutant Trx m/affinity trapping IEF/SDS 2DE	43	Balmer et al., 2006b [104]
Algae and cyanobacteria			
Chlamydomonas reinhardtii (Autotrophic growth)	Mutant Trx h/affinity trapping SDS-PAGE	1	Goyer et al., 2002 [89]
(Growth on acetate)	Trx h/affinity trapping IEF/SDS 2DE	55	Lemaire et al., 2004 [91]
Synechocystis			
Soluble and peripheral proteins	*Synechocystis* mutant Trx A/affinity trapping SDS-PAGE Non-reducing/reducing SDS 2DE	25	Lindahl and Florencio, 2003 [90]
Cytosol	*Synechocystis* mutant Trx A/affinity trapping SDS-PAGE	5	Hosoya-Matsuda et al., 2005 [92]
	Synechocystis mutant Trx A, B and Q/affinity trapping SDS-PAGE Non-reducing/reducing SDS 2DE	14 new	Perez-Perez et al., 2006 [93]
Total membranes	*Synechocystis* mutant Trx A *In situ* binding to His-tagged mutant Trx A /Ni-Affinity trapping Non-reducing/reducing SDS 2DE	50	Mata-Cabana et al., 2007 [109]

Each number corresponds to proteins or protein subunits having different names.

of glucose 6-phosphate dehydrogenase, a member of the oxidative pentose phosphate pathway [42]. Details for relating these and other enzymes to Trx in the preproteomics era are given elsewhere [1,10,34]. Here, suffice it to say that during this period, several other key plastid enzymes were found to be activated by Trx: acetyl-CoA carboxylase [45], RubisCO activase [46], and ADP-glucose pyrophosphorylase [47]. These findings extended the role of Trx to the regulation of pathways of fatty acid and starch synthesis. Further, with the inclusion of RubisCO activase, Trx emerged as a regulator of each of the five control points in the Calvin–Benson cycle.

The original experiments linking Trx to particular enzymes were based on changes in their activity observed after reduction, typically with dithiothreitol (DTT). In most cases, the early studies have been confirmed and extended by other lines of evidence, typically mutagenesis experiments [1]. Ensuing findings gave credence to the view expressed early on that Trx enables the chloroplast to minimize so-called futile cycles and maximize use of available energy resources [38,48–50]. With time, Trx emerged as a "biochemical eye" for the plastid, enabling the functional separation of diurnal and nocturnal processes [1,10].

After the discovery of two forms of Trx in chloroplasts, *f* and *m* (named after their capability to reduce FBPase and MDH, respectively), a third type of Trx was found outside the plastid, notably in the cytoplasm, endoplasmic reticulum and mitochondria. Biochemical studies initiated with wheat shortly thereafter provided evidence that the extraplastidic isoform (designated Trx h for heterotrophic) regulates germination, seedling growth and development. Localized in the starchy endosperm, Trx h was concluded to act through its reductive capability in three ways, i.e., by: (1) increasing the solubility and susceptibility of storage proteins to proteases [51–55] (2) directly activating enzymes such as thiocalsin, a protease specific for the reduced forms of gliadins and glutenins [56]; and (3) inactivating proteins that inhibit proteases [57,58], α-amylase [59] and starch debranching enzyme (pullulanase) [60]. In this way, Trx promotes the mobilization of the protein and starch reserves of the seed. The biochemical studies were confirmed and extended by subsequent genetic engineering experiments with barley. Overexpression of Trx h targeted to the protein body enhanced the activity of pullulanase and accelerated germination in concert with early onset of α-amylase activity [61,62].

In parallel studies, Trx *h* isoforms have also been found to play a role in a self-incompatibility mechanism leading to self-pollen rejection [63–65]. A role of Trx in other biotic interactions of plants with pathogenic or symbiotic organisms has since been demonstrated [26, 66–68].

4. Thioredoxin targets identified using proteomic approaches

The past decade has witnessed the introduction of large-scale investigations of biological systems. Genomic analysis, in particular assembly of complete genome sequences and compilation of ESTs (expressed sequence tags), has provided an invaluable foundation for these investigations. The translation of this wealth of genomic data into functional analysis of cellular processes is dependent on the characterization of the proteome it encodes. The development of more sensitive Edman microsequencing methods, refinements in sensitivity and accuracy of mass spectrometry instrumentation, and improvements in software for interrogating the rapidly growing genome and EST databases to predict protein sequences have made high-throughput analysis of protein populations possible. A major challenge in proteomic studies has been the identification of low abundance proteins in eukaryotic cells. The development of protein labeling techniques and affinity chromatography methods has enabled the detection and isolation of fractions enriched in putative Trx targets (Table 1). A proteomic approach in which the Trx targets are separated by electrophoresis in one- or two-dimensional-gels (SDS-PAGE or 2DE) and identified by mass spectrometry has made it possible in the last 7 years to find specific cellular processes regulated by this protein class. A recent application of a labeling technique based on the use of isotope-coded affinity tags (ICAT), moreover, has provided a new approach not only to identify Trx targets, but also to determine their redox-active disulfides. The potential and established Trx targets identified in the past 30 years are listed in Table 2.

4.1. Targets labeled with thiol specific probes

The strategy of identifying potential targets by labeling sulfhydryl groups newly generated via Trx reduction was devised by Yano et al. [69] and originally applied to proteins extracted from dry peanut seeds (Fig. 2). Disulfide bonds of proteins in a crude extract were reduced *in vitro* with a reconstituted NTS consisting of a *C. reinhardtii* Trx *h*, an *Arabidopsis* NTR and NADPH. The newly generated sulfhydryl groups were visualized using monobromobimane (mBBr)—a thiol-specific probe that fluoresces after covalently reacting with an SH group. Earlier, mBBr was employed to demonstrate that Trx reduced storage proteins of cereal seeds [52,57,58]. Peanut proteins were then separated by non-reducing/reducing SDS 2DE (no reducing agent in the first dimension and mercaptoethanol in the second dimension) to determine the type of bond reduced. Proteins without disulfide bonds migrated along a diagonal line, while those with intramolecular disulfide bridges migrated above the diagonal line and multi-subunit proteins with intermolecular disulfide bridges migrated below the diagonal line (Fig. 2). The

Table 2 – Potential and established targets of Trx in land plants and oxygenic photosynthetic microorganisms.

Land plants
Acetyl-CoA carboxylase [1,85,87,104]
Acetylornithine aminotransferase [104]
Aconitase [88,100]
Actin [100,106]
Acyl-[acyl-carrier protein] thioesterase [104]
Adenosine kinase 1 [75]
Adenosylhomocysteinase [75]
S-Adenosylmethionine synthetase [100]
S-Adenosylmethionine synthetase 1 [75]
Adenylate kinase [88]
Adenylosuccinate synthetase [104]
ADP-glucose pyrophosphorylase [1,72,87]
ADP-glucose pyrophosphorylase, LS [100,104]
ADP-glucose pyrophosphorylase, SS [104]
ADP-ribosyltransferase (post maturation protein 38) [106]
Alanine aminotransferase [72,74,88,100,105]
Alanine aminotransferase 2 [75]
Alanine aminotransferase, putative [74,105]
2S albumin [53,106]
Alcohol dehydrogenase [86]
Alcohol dehydrogenase 1 [75]
Alcohol dehydrogenase short chain [106]
Alcohol dehydrogenase CPRD12 [88]
Aldehyde dehydrogenase [88,100,106]
Aldehyde oxidase [100]
Aldo/keto auxin induced reductase [106]
Aldose 1 epimerase [106]
Aldose reductase
Allene oxide cyclase [88]
Allergen 28 K [106]
Allyl alcohol dehydrogenase
Alternative oxidase [22]
Aluminum-induced protein-like [75]
Aminotransferase AGD2, putative [104]
α-Amylase [130]
α-Amylase inhibitor 0.19 [72,100]
α-Amylase inhibitor 0.28 [100]
α–Amylase inhibitor 0.53 [100]
α-Amylase inhibitor Ima 1 [100]
α-Amylase inhibitor BDAI-I [73,75]
α-Amylase inhibitor BMAI-1 [73]
α-Amylase/chymotrypsin inhibitor WCI [100]
α-Amylase/subtilisin inhibitor [72]
α-Amylase/subtilisin inhibitor (BASI) [73]
α-Amylase/subtilisin inhibitor CMa [73]
α-Amylase/subtilisin inhibitor CMb [73]
α-Amylase/subtilisin inhibitor CMd [73]
α-Amylase/subtilisin inhibitor CMe [73]
α-Amylase/subtilisin inhibitor (WASI) [100]
α-Amylase/trypsin inhibitor CM3 [100]
α-Amylase/trypsin inhibitor CM16 [100]
α-Amylase/trypsin inhibitor CM17 [100]
α-Amylase/trypsin inhibitor pUP13 [73]
β-Amylase (chloroplast) [1,75,85]
Apospory-associated protein C-like [74,105]
Arginine methyltransferase [106]
Argininosuccinate lyase [75,104]
Ascorbate peroxidase [75,100]
Ascorbate peroxidase 1 (cytosol) [74,86,105]
Aspartate aminotransferase [75,88]
Aspartate-semialdehyde dehydrogenase [75]
ATPase subunit α (vacuole) [106]
ATP dependent clp protease ATP-binding subunit [85,87,104]

Table 2 (continued)

Land plants

ATP-dependent DNA helicase [85]
ATP sulfurylase [105]
ATP synthase (chloroplast) [1]
ATP synthase subunit α (chloroplast) [87,98,106]
ATP synthase subunit β (chloroplast) [98,107]
ATP synthase subunit γ (chloroplast) [87,107]
ATP synthase subunit γ chain 1 (chloroplast) [96]
ATP synthase subunit ε (chloroplast) [98]
ATP synthase subunit α (mitochondrium) [88]
ATP synthase subunit β (mitochondrium) [88,100,106]
ATP synthase subunit δ (mitochondrium) [88]
Auxin-induced protein [100]
Avenin [72,100]
Avenin N9 [100]
Avenin-like a precursor, putative [75]
Barwin
Barperm1 [75]
Betaine aldehyde dehydrogenase [75]
B1 Hordein [75]
Bowman-Birk type trypsin inhibitor [75]
Branched-chain keto acid decarboxylase subunit E1b [88]
Brittle-1 protein (ADP glucose transporter, plasts) [104]
Caffeoyl-CoA O-methyltransferase [75]
Calreticulin [75,106]
Carbonic anhydrase (chloroplast) [74,85,87,105]
Carbonic anhydrase (cytosol) [74,105]
Catalase [88,100,106]
Catalase 2 [105]
Cell division cycle protein CDC48-homolog [75,100,106]
Chalcone reductase [106]
Chaperonin 60 kDa (chloroplast) [87]
Chaperonin 60 kDa subunit α (chloroplast) [85,86,104,106]
Chaperonin 60 kDa subunit β (chloroplast) [85,86,100,104,106]
Chaperonin 60 kDa (mitochondrium) [88]
Chaperonin 60 kDa subunit 2 (mitochondrium) [106]
Chitinase, homolog to wheat chitinase 3 [73]
Chlorophyll a/b binding protein (LHCIIb) [107]
Chlorophyll a/b binding protein CP26 (LHCB5) [98]
Chloroplastic nucleoid DNA binding protein [74,105]
Chloroplastic protein CP12 [74,105]
Chloroplastic protein CP12-like [74,105]
Cinnamoyl-CoA reductase [106]
CoA-thioester hydrolase [88]
Cold shock protein-1 [75]
Conglutin [106]
4-Coumarate-CoA ligase [106]
Cyclophilin [1,73,74,82,105]
Cyclophilin A [72,100]
Cyclophilin-like [104]
Cyclophilin A2 [75]
Cysteine protease AALP [74,105]
Cysteine synthase [85,88]
Cytochrome c oxidase subunit 5b [88]
Cytochrome c oxidase subunit 6b [88]
Cytochrome f (PetA) [98]
Dehydrin [106]
Dehydroascorbate reductase [75]
Dehydroascorbate reductase (GSH) [72,74,100,105]
1-Deoxyxylulose 5-phosphate reductoisomerase [85]
3-Deoxyarabinoheptulosonate 7-phosphate synthase [1]
Desiccation-related protein [69,100]
Dienelactone hydrolase [112]
Dihydrolipoamide acetyltransferase [88]
Dihydrolipoamide dehydrogenase [88]
Dihydrolipoyl dehydrogenase [75]

Table 2 (continued)

Land plants

Dihydroorotate dehydrogenase, putative [75]
Dihydroxyacid dehydratase [74,104,105]
DNA polymerase d auxiliary protein [106]
Electron transport chain (chloroplast) [1]
Elongation factor 1 subunit α [106]
Elongation factor 1 subunit β [100,106]
Elongation factor 1 subunit δ [106]
Elongation factor 2 [86,100,106]
Elongation factor G [85]
Elongation factor Tu [85,87,88,106]
Embryo-specific protein [73,106]
Embryo-specific protein 2 [112]
Embryo-specific protein Ose731, putative [75]
26 kDa Endochitinase 1 [73]
26 kDa Endochitinase 2 [73]
Endoplasmin-homolog [104]
Enolase [72,75,85,100,106]
Enolase, putative [104]
Erv1 and Erv2-related protein (chloroplast) [108]
Eukaryotic translation initiation factor eIF4A [86]
Eukaryotic translation initiation factor 5A1 [75]
Eukaryotic translation initiation factor 5A2 [106]
33.3 kDa Expressed protein (F24 J8.7) [105]
Ferredoxin 1 [74,105]
Ferredoxin 2 [74,105]
Ferredoxin-thioredoxin reductase [1]
Ferredoxin nitrite reductase [74,105]
Ferritin [106]
FKBP peptidylprolyl cis-trans isomerase (cytosol) [106]
FKBP peptidylprolyl cis–trans isomerase (chloroplast) [1,74,105]
Formate dehydrogenase [105]
Formate dehydrogenase (mitochondria) [88,100]
10-Formyltetrahydrofolate synthase [100]
Fructose 1,6-bisphosphatase [1,87]
Fructose 1,6-bisphosphatase, putative [74,105]
Fructose 1,6-bisphosphatase 2 [75]
Fructose 1,6-bisphosphate aldolase [72,100,104,107]
Fructose 1,6-bisphosphate aldolase, plastid [96]
Fructose 1,6-bisphosphate aldolase-like [74,86,105]
Fructose 1,6-bisphosphate aldolase, putative [105]
FTSH2 [98]
FTSH8 [98]
FtsZ protein (plastid division) [85]
GcpE protein (Isoprenoid biosynthesis) [85]
Germin-like [86]
Gliadin
Globulin [100]
Globulin (putative) [112]
Globulin 1, barley embryo (Beg1) [70]
Globulin, embryo [75]
Globulin, seed [72]
Globulin 1S-like [112]
α-1,4-Glucan phosphorylase [104]
α-Glucan, water dikinase [1]
Glucose and ribitol dehydrogenase [75]
Glucose-6-phosphate dehydrogenase (chloroplast) [1]
Glucose-6-phosphate isomerase [104]
β-glucosidase
Glutamate dehydrogenase [88]
Glutamate 1-semialdehyde 2,1-aminomutase [85,87]
Glutamate synthase GOGAT (Fdx; chloroplast) [1,104]
Glutamine synthase (chloroplast) [74,82,85–87,105]
Glutamine synthase (cytosol) [106]
Glutaredoxin [75]

Table 2 (continued)

Land plants

Glutaredoxin-like [88]
Glutathione peroxidase [100,106]
Glutathione-S-transferase (mitochondrium) [106]
Glutathione-S-transferase (phi class) [74,105]
Glutathione-S-transferase (tau class) [105]
Glutenin LMW Glutenin subunit [100]
LMW Glutenin subunit b [72]
Glyceraldehyde-3-phosphate dehydrogenase [75]
Glyceraldehyde-3-phosphate dehydrogenase (NAD) [72,73,86,100,106]
Glyceraldehyde-3-phosphate dehydrogenase (NADP) [1,74,82,85,87,105]
Glycerate kinase [131]
Glycine cleavage system protein H [74,88,105]
Glycine cleavage system protein H, probable [105]
Glycine cleavage system protein P [88]
Glycine cleavage system protein P-like [105]
Glycine cleavage system protein T [88]
Glycinin basic subunit, allergen Ara h3 [69]
Glycinin subunit G7 [106]
Glycine-rich protein [106]
Glycyl-tRNA synthetase [75]
Glyoxalase [72]
Glyoxalase-like (single domain) [73]
GTP-binding protein [87,105,106]
Guanine nucleotide-binding protein subunit β [100,106]
Heat shock protein ClpB, putative [104]
Heat shock protein 70 kDa [100,104]
Heat shock protein 70 kDa (cytosol) [106]
Heat shock protein 70 kDa (chloroplast) [85-87,106]
Heat shock protein 70 kDa (endoplasmic reticulum) [106]
Heat shock protein 70 kDa, Dna-K molecular chaperone [88]
Heat shock protein 70 kDa (mitochondrium) [106]
Heat shock protein 82 kDa [104]
Heat shock protein 83 kDa [106]
Heat shock protein 90 kDa [106]
Heat shock protein 90 kDa, putative [106]
Hypothetical protein (O65502) [107]
Hypothetical protein (gi|147769189|) [75]
Hypothetical protein (19.9 kDa; Q9ZPZ4) [74,105]
Hypothetical protein (29.8 kDa) [88]
Hypothetical protein (36.2 kDa) [88]
IN2-1 protein [75]
Imidazole glycerol phosphate synthase hisHF [104]
Inorganic pyrophosphatase (chloroplast) [104]
Inorganic pyrophosphatase (soluble) [75]
Isocitrate dehydrogenase [88]
Isocitrate dehydrogenase (NADP; cytosol) [74,105]
Isopropylmalate dehydratase [106]
3-isopropylmalate dehydratase (small subunit) [104]
Isovaleryl-CoA dehydrogenase [88]
Ketol-acid reductoisomerase [75,106]
Ketol-acid reductoisomerase, putative [104]
Late embryogenesis abundant protein, LEA of group 5 [106]
Legumin A [106]
Legumin J [106]
Leucine aminopeptidase [88,106]
Leucine aminopeptidase, putative [104]
Leukotriene-A4 hydrolase like [106]
Light harvesting complex II protein kinase [1]
Lipid transfer protein [75]
Lipid transfer protein 7a2b [75]
Lipoamide dehydrogenase [74,105]
Lipoamide dehydrogenase, putative [104]

Table 2 (continued)

Land plants

Lipoxygenase [106]
Magnesium chelatase [85]
Malate dehydrogenase (NAD) [72,86,100]
Malate dehydrogenase (NAD, cytosol) [74,105,106]
Malate dehydrogenase (NAD, mitochondrium) [88,105]
Malate dehydrogenase (NADP, chloroplast) [1,74,85,87,105,108]
Malic enzyme [88]
Malic enzyme (cytosol) [106]
Mannitol dehydrogenase [74,105]
Mercaptopyruvate sulfurtransferase [88]
MERI 5 protein (xyloglucan endotransglycolase) [105]
Methionine sulfoxide reductase A [132]
Methionine sulfoxide reductase B (chloroplast) [96,132]
Peptide methionine sulfoxide reductase [74,105]
Methionine synthase [100]
Methionine synthase (VitB12 independent) [86]
5,10-Methylene tetrahydrofolate dehydrogenase [106]
4-Methyl-5-(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein [106]
Methylmalonate-semialdehyde dehydrogenase [88]
Monodehydroascorbate reductase [75]
Monogalactosyldiacyglycerol synthase [1]
Myrosinase [74,105]
NADH-ubiquinone oxidoreductase 75 kDa subunit [88]
NADP-thioredoxin reductase
NADPH:protochlorophyllide oxidoreductase translocon protein PTC52, precursor [108]
p-Nitrophenylphosphatase [105]
Ni-binding urease accessory protein [106]
Non-specific lipid transfer protein [106]
Non-specific lipid transfer protein 1 [73,75]
Non-specific lipid transfer protein 6 [75]
Non-specific lipid-transfer protein precursor [100]
Nuclear transport factor 2 [75]
Nucleoside diphosphate kinase [88]
Ornithine carbamoyltransferase, putative [104]
Os01 g0611000 [75]
Os03 g0277300 [75]
Os03 g0685500 [75]
Os03 g0734100 [75]
Os03 g0794700 [75]
Os05 g0155100 [75]
Os05 g0489200 [75]
Os06 g0134800 [75]
Os06 g0538000 [75]
Os12 g0235800 [75]
Os12 g0567700 [75]
Oxidoreductase, zinc-binding dehydrogenase [75]
Oxygen evolving enhancer OEE1 [107]
Oxygen evolving enhancer OEE1-1 [74,105]
Oxygen evolving enhancer OEE1-2 [74,105]
Oxygen evolving enhancer OEE 2 [107]
Oxygen evolving enhancer OEE 2-1 [105]
P0460c04.20 [100]
Papain [133]
Pathogenesis-related protein 5 (thaumatin) [74,105]
Peroxidase [72,100]
Peroxiredoxin [88]
1-Cys Peroxiredoxin [70,72,73,75,100,106]
2-Cys Peroxiredoxin [1,82,85,100]
2-Cys Peroxiredoxin BAS1 [95,96,104–106]
Peroxiredoxin II [74,86,105]
Peroxiredoxin II (cytosol) [106]
Peroxiredoxin IIE (chloroplast) [106]

Table 2 (continued)
Land plants

Peroxiredoxin IIF (mitochondria) [106]
Peroxiredoxin-like [105]
Peroxiredoxin Q [1,82,96]
Peroxiredoxin Q-like [82,105]
Peroxiredoxin TPX1 [105]
Phenylalanine ammonia lyase [134]
Pheophorbide a oxygenase (lethal leaf spot protein LLS1) [108]
Phosphoenolpyruvate carboxylase protein kinase [135]
6-Phosphogluconate dehydrogenase [85]
Phosphoglycerate dehydrogenase [85,106]
Phosphoglycerate dehydrogenase, putative [104]
Phosphoglycerate kinase [87,104,105]
Phosphoglycerate mutase [100,106]
Phosphoglycerate mutase (2,3-bisphosphate-independent) [75]
Phospholipid hydroperoxide glutathione reductase [88]
Phosphoribulokinase [1,74,85,105,108]
Photosystem I reaction center subunit N [98,107]
Photosystem I reaction center subunit psaK [107]
Plastocyanin [105,107]
Poly(A)-binding protein [100]
Porin (VDAC) [88]
Post maturation protein 24 [106]
Post maturation protein 25 [106]
Post maturation protein 28 [106]
Post maturation protein 34 [106]
Post maturation protein 51 [106]
Profilin [100]
Proliferating cell nuclear antigen [75]
26S Proteasome regulatory subunit RPN12 [86]
26S Proteasome regulating subunit S6 [72]
26S Proteasome regulating subunit S6A [106]
26S Proteasome regulatory subunit S12 [100]
5a2 Protein [75]
14-3-3 Protein [106]
Protein, putative (At5 g10860.1) [88]
2S protein, allergen Arah2 [69]
2S protein, allergen Arah6 [69]
Protein disulfide isomerase [72,74,100,104–106]
Protein disulfide isomerase-like [106]
Protein phosphatase 2A regulatory subunit A [75]
Protein translocon protein TIC55 [108]
Purothionin [51]
Pyrophosphate fructose 6-phosphate 1 phosphotransferase [72,100]
Pyruvate decarboxylase [106]
Pyruvate dehydrogenase subunit E1α [88,104]
Pyruvate dehydrogenase subunit E1β [88]
Pyruvate kinase-like [75]
Pyruvate phosphate dikinase [72,100]
Ran (Ras-related nuclear protein) [106]
Ran-binding protein [106]
Ras-related protein RIC1 [75]
Retrotransposon protein [75]
Reversibly glycosylated polypeptide [72,75,100]
Ribonuclease-like protein 1, regulator [100]
28 kDa Ribonucleoprotein [85,87]
Ribose 5-phosphate isomerase [74,105]
Ribosomal protein L3 [75]
Ribosomal protein L4 [87]
Ribosomal protein L10A [75]
Ribosomal protein L21 [87]
Ribosomal protein L32 [75]
Ribosomal protein S1 [87]
Ribosomal protein S5 [87]
Ribosomal protein S6 (PrpS6) [85]

Table 2 (continued)
Land plants

Ribosomal protein S11 [75]
Ribosomal protein S12 [100]
Ribosomal protein S30 [87]
Ribosomal protein SR
30S Ribosomal protein S1 [85]
40S Ribosomal protein S3a [75]
40S Ribosomal protein S3-1 [75]
40S Ribosomal protein S5 [75]
40S Ribosomal protein S6 [75]
40S Ribosomal protein S12 [75]
40S Ribosomal protein S21 [106]
40S Ribosomal protein S26 [75]
40S Ribosomal protein S27a [75]
60S Ribosomal protein, putative [75]
60S Ribosomal protein L4 [75]
60S Ribosomal protein L10 [75]
60S Ribosomal protein L10a-1 [75]
60S Ribosomal protein L17-1 [75]
60S Ribosomal protein L21 [75]
60S Ribosomal protein L22-2 [75]
60S Ribosomal protein L28, putative [75]
60S acidic Ribosomal protein P^3 [70]
Ribulose 5-phosphate 3-epimerase [74,85,105]
Rieske FeS protein [98,107]
Ripening-related protein [106]
RNA-binding protein [105,106]
RNA-binding protein 24 kDa [87]
RNA-binding protein 41 kDa [87]
RNA-binding protein (glycine-rich) [100,106]
mRNA binding protein [105]
RubisCO activase [1,74,82,85,87,105,107]
RubisCO small subunit [82,85,87,96,105,107]
RubisCO large subunit [87,104–107]
Sali 3.2 [106]
Sedoheptulose 1,7-bisphosphatase [1,74,82,85,105,108]
Seed biotinylated protein SBP 65 [106]
Seed maturation protein [69]
Selenium-binding protein [106]
Serine hydroxymethyltransferase [88,104,105]
Serpin [72,100]
Seryl-tRNA synthetase 1 [75]
S-locus receptor kinase (self incompatibility mechanism) [64,65]
Sorbitol 6-phosphate dehydrogenase [106]
SOUL-heme-binding protein [106]
Spermidine synthase 1 [75]
S-RNAse [23]
Starch branching enzyme IIa [104]
Stress-induced protein Sti [106]
Stress induced protein Sti-1 like [100]
Subtilisin/chymotrypsin inhibitor
Succinate dehydrogenase [106]
Succinate dehydrogenase (flavoprotein subunit) [88]
Succinic semialdehyde dehydrogenase [106]
Succinyl-CoA ligase subunit α1 [75]
Succinyl-CoA ligase subunit α [88]
Succinyl-CoA ligase subunit β [88]
Sucrose binding protein P54 [106]
Sucrose non fermenting-related kinase subunit 4b [106]
Sucrose:fructan 6-fructosyltransferase [75]
Sucrose synthase 1 [75]
Sulfite reductase [100]
Superoxide dismutase (fragment) [73]
Superoxide dismutase (Cu–Zn) [100]
Superoxide dismutase (Cu–Zn) (chloroplasts) [73]
Superoxide dismutase (Mn) [88]

Table 2 *(continued)*

Land plants

T-complex protein CPN60 chaperonin ε [106]
T-complex protein CPN60 chaperonin τ [106]
Thaumatopain [133]
Thiamin biosynthesis protein [85]
Thiamin biosynthesis protein ThiC [104]
Thiazole biosynthetic enzyme [85]
Thiocalsine [56]
Thiosulfate sulfurtransferase [104]
Threonine synthase [104]
Transcriptional regulator [96]
Transketolase [74,85,87,104,105]
Triose phosphate isomerase [72,100]
Triose phosphate isomerase (chloroplast) [74,85,105]
Triose phosphate isomerase (cytosol) [105,106]
Triticin [100]
Trypsin inhibitor CMe [73]
Tryptophan synthase β-chain 1 [104]
Tubulin chain α [106]
Tubulin chain α-3 [100]
Tubulin chain β [106]
Tubulin chain β-3 [100]
17.4 kDa Thylakoid luminal protein [74,105]
Tyrosine transaminase-like [105]
Ubiquinol-cytochrome c reductase subunit II [88]
Ubiquitin conjugating enzyme E2 [100]
Ubiquitin-specific protease 14 (UBP14) [75]
UDP-glucose dehydrogenase [106]
UDP-glucose pyrophosphorylase [100,106]
Unknown function protein (Q9SGT3) [85]
Unknown protein 1 (TC101829) [106]
Unknown protein 2 (TC97992) [106]
Unknown protein 3 (TC108880) [106]
Unknown protein 4 (TC109465) [106]
Unknown protein 5 (TC106727) [106]
Uroporphyrinogen decarboxylase [85,105]
Vicilin 47 kDa (whole protein and fragments) [106]
Wali 7 [106]
WD-repeat protein RBPA1 [100]

Chlamydomonas and other green algae
Acetyl-CoA synthetase [91]
Aconitase [91]
Actin [91]
Adenylate kinase [91]
S-Adenosylmethionine synthetase [91]
5'-Adenylylsulfate reductase [91]
AIR synthase [91]
Aldose 1 epimerase-like [91]
Argininosuccinate synthetase [91]
ATPase subunit α (vacuole) [91]
ATPase subunit E (vacuole) [91]
ATP synthase subunit α (chloroplast) [91]
Catalase [91]
Chaperonin 20 kDa [91]
Chaperonin 60 kDa subunit α [91]
Diaminopimelate epimerase [91]
Dihydrolipoamide succinyltransferase (oxoglutarate
dehydrogenase complex su E2) [91]
Dihydroxyacid dehydratase [91]
Elongation factor 1 [91]
Elongation factor 2 [91]
Elongation factor Tu [91]
Enolase [91]
Ferredoxin [91]

Table 2 *(continued)*

Land plants

Fructose 1,6-bisphosphate aldolase (chloroplast) [91]
Glutamine synthase (chloroplast) [1,91]
Glutamine synthase (cytosol) [91]
Glutathione peroxidase [91]
Heat shock protein 70 kDa HSP 70B [91]
Heat shock protein 70 kDa HSP 70A [91]
Hypothetical protein (9270) [91]
Isocitrate lyase [91]
Inorganic pyrophosphatase [91]
Inorganic pyrophosphatase (soluble) [91]
Isopropylmalate dehydrogenase [91]
Ketol acid reductoisomerase [91]
LytB [91]
Malonyl-CoA ACP transacylase [91]
Peptide methionine sulfoxide reductase [91]
NiFe-hydrogenase [1]
Oxygen evolving enhancer OEE1 [91]
2-Cys Peroxidase [89]
2-Cys Peroxiredoxin (cytosol) [91]
2-Cys Peroxiredoxin (chloroplast) [91]
Peroxiredoxin IIE [91]
Phosphoribulokinase [91]
26S Proteasome particle 12 [91]
Protein containing rhodanese domain [91]
Psba mRNA(encoding the PS II D1 protein)-binding protein [1]
Ran (Ras-related nuclear protein) [91]
RB60 protein disulfide isomerase [1,91]
Reversibly glycosylated polypeptide [91]
Ribose 5-phosphate isomerase [91]
RubisCO small subunit [91]
RubisCO large subunit [91]
Sedoheptulose 1,7-bisphosphatase [91]
Serine protease-like [91]
Thiazole biosynthetic enzyme [91]
Threonine synthase [91]

Cyanobacteria
Acetolactate synthase, small subunit [109]
ADP-glucose pyrophosphorylase [90]
Allophycocyanin β [92]
Argininosuccinate lyase [93]
Argininosuccinate synthetase [90]
Aspartyl-tRNA synthetase [109]
ATPase of the AAA-family [109]
ATP synthase, subunit α [109]
ATP synthase, subunit β [109]
Carbonic anhydrase, type β [109]
Carboxysomal protein [90,109]
Catalase-peroxidase [93]
Chaperonin 1 60 kDa GroEL [90,109]
ClpB1 [109]
ClpC [109]
Elongation factor G [90]
Elongation factor Tu [90,109]
Ferredoxin sulfite reductase [90,109]
Fructose 1,6-bisphosphate aldolase, class II [93]
FtsH [109]
GDP-mannose dehydratase [90]
Glutamate synthase GOGAT (Fdx) [90]
Glutamate synthase GOGAT (NADH) [90]
Glucan branching enzyme [90]
Glyceraldehyde 3-phosphate dehydrogenase 2 [93,109]
Glycogen phosphorylase [93]
Glycogen synthase 2 [90]

Table 2 (continued)

Land plants

GST [109]
GTP-binding protein, putative [109]
Heme oxygenase 1 [109]
Hypothetical protein (sll1106) [109]
Hypothetical protein (slr0658) [109]
Hypothetical protein (slr1702) [109]
Hypothetical protein (slr1855) [93]
Hypothetical protein ycf50 (slr2073) [109]
Isocitrate dehydrogenase (NADP) [136]
Lysyl-tRNA synthetase [93]
Type 2 NADH dehydrogenase, NdbC [109]
Nitrate/nitrite transporter, ATP binding subunit (NtrD) [109]
Oxyanion-translocating ATPase, ArsA [109]
PAPS sulfotransferase [137]
1-Cys Peroxiredoxin [90,92,109]
Peroxiredoxin II (YLR109-homolog) [90,92]
Phosphoglucomutase [90]
Phosphoglycerate kinase [93]
Phosphoribulokinase [93,109]
Photosystem I protein PsaD [109]
Phycobilisome core linker (L_c) [90]
Phycobilisome core-membrane linker (L_{CM}) [90,109]
Phycobilisome rod-core linker polypeptide (L_{RC}) [109]
Phycocyanin subunit β [92,93,109]
Polyribonucleotide nucleotidyltransferase [93]
Polyribonucleotide nucleotidyltransferase subunit a [109]
Porphobilinogen synthase [90]
Precorrin isomerase [109]
Preprotein translocase SecA [109]
Pyruvate dehydrogenase component E1, subunit α [109]
Pyruvate dehydrogenase component E1, subunit β [109]
Pyruvate dehydrogenase component E2 [93,109]
RecA [109]
Response regulator Rre1 [109]
30S Ribosomal protein S2 [109]
30S Ribosomal protein S3 [90,109]
50S Ribosomal protein L3 [109]
RNA polymerase, subunit α [109]
RNA polymerase subunit β [90]
RNA polymerase subunit β' [90]
RubisCO large subunit [90,109]
Serine-O-acetyl transferase [109]
Sugar-nucleotide epimerase [90]
Sulfate adenylyltransferase [90]
Thioredoxin A [93]
Transcription antitermination protein NusG [109]
Transcription termination factxor NusA [109]
Transketolase [93]
Twitching motility protein PilT [109]
Universal stress protein-family, Usp1 [109]
Universal stress protein-family, Usp2 [109]
Unknown protein (ssl2245) [92]
Urea transporter, ATP-binding subunit (UrtD) [109]
Valyl-tRNA synthetase [90]

The list corresponds to an updated version of the list published in 2005 by Buchanan and Balmer [10]. Several of these proteins have been previously described in this review and by Schürmann and Buchanan [1]. Only established targets identified in biochemical studies and potential targets recovered in screens using native and mutant Trxs or Trx-like proteins, whether validated or not, are listed. Potential redox regulated proteins recovered in other screens for disulfide proteins or using mutant Grx are not included.

study demonstrated that NTS was effective in protein reduction and revealed, as expected, a preference for proteins with intramolecular disulfide bonds, confirming earlier results with castor seed 2S albumin [53]. In contrast, other cell redox systems utilizing GSH or Grx were consistently without effect as shown previously with the 2S albumin and other storage proteins. The labeled proteins were also separated by IEF/SDS 2DE and 20 spots were selected for identification by Edman sequencing of N-terminal or internal peptides. Proteins in five of these spots were identified—three were related to storage proteins (two 2S proteins and glycinin B), one to a desiccation-related protein and another to a seed maturation protein. The combination of 2DE separation of mBBr-labeled proteins with polypeptide sequencing was next used to identify Trx targets in embryos of germinating barley grain [70]. Among the proteins identified was Beg 1 (barley embryo globulin 1). An increase in solubility of the albumin and globulin storage proteins was also observed following seed imbibition. This was interpreted to result from a combination of reduction and partial hydrolysis of these proteins that accompanied germination. A Prx (1-Cys Prx), and acidic ribosomal protein P^3 were also found in the screen, thereby linking Trx h to protection against ROS and translation. It is noted that Trx was previously demonstrated to intervene in the regulation of translation of the D1 protein, of the photosystem II reaction center in *C. reindhardtii* chloroplasts, through the reduction of a specific disulfide in the PsbA mRNA binding protein complex [71].

The fluorescent thiol probe method was next applied to mature wheat grain using the *E. coli* Trx redox system that, while more efficient, led to results similar to those obtained with the homologous system [72]. Twenty-three Trx-linked endosperm proteins were identified, 12 not recognized as targets at the time. The proteins function in a spectrum of enzymes of key processes (metabolism, protein storage, protein degradation, or protein assembly/folding), extending the role of Trx in the endosperm. Enzymes functional in cell metabolism that were validated by biochemical studies included cytosolic homologs of GAPDH and MDH, whose activity is dependent on NAD. The effect of reduction on the cytosolic isoforms of these enzymes was not known, but *in vitro* Trx reduction increased activity [72]. By contrast, using site-directed mutagenesis, Ruelland et al. [49] earlier identified primary sequence extensions that contain conserved Cys capable of conferring redox regulation to the NADP-linked form of MDH found in chloroplast.

Maeda et al. [73] built on the fluorescent gel approach and substituted a higher sensitivity fluorescent dye, cyanine 5 maleimide (Cy5m), for mBBr to visualize newly generated sulfhydryl groups. Using homologous NTR and two different isoforms of Trxs h that gave similar results, they identified 16 different proteins targeted by Trx h in dry and germinating barley seeds. Among them were several isoforms of α-amylase/subtilisin inhibitors and cyclophilin, both known Trx targets, and previously unrecognized targets—chitinase, endochitinase, embryo-specific protein (ESP), lipid transfer protein, glyoxalase-like protein, and superoxide dismutase. Comparison of proteins labeled with Cy5m and mBBr revealed that the methods are complementary in that different target proteins were identified with each dye. Notably, the use of mBBr led to the identification of four additional potential targets. Interestingly, Maeda et al. [73] found that GAPDH and 1-Cys Prx were labeled with mBBr
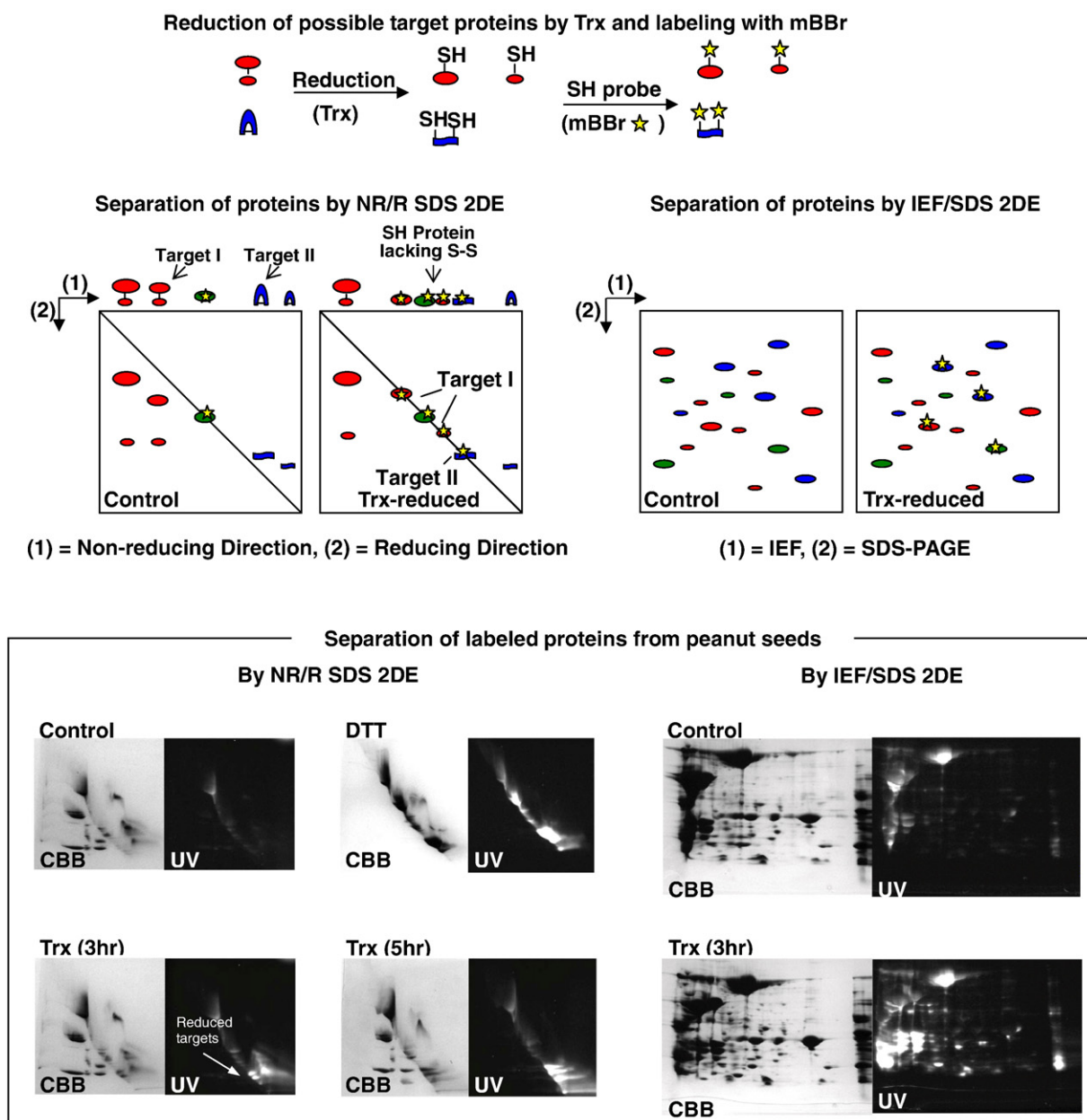
Fig. 2 – Fluorescent labeling of Trx target proteins with mBBr followed by separation in 2-D gels. Top: Fluorescent labeling with mBBr of target proteins reduced by Trx or DTT, followed by separation by non-reducing/reducing SDS 2DE, or IEF/SDS 2DE. Bottom: Application using peanut proteins. Fluorescent proteins were observed under UV light (UV; dark panels) before the staining of all proteins by Coomassie brilliant blue (CBB; grey panels). From [69] with modification. Copyright (2001) National Academy of Sciences, U.S.

but not with Cy5m. They suggested that the two dyes differ in ability to access buried thiol groups or that Cy5m labeling might have shifted the pIs of these enzymes such that they co-migrated with other proteins. Maeda et al. also noted that few barley seed target proteins corresponded to those identified previously in wheat endosperm by Wong et al. [72]. They suggested that this was due to experimental conditions—Trx from different sources was used (*E. coli* Trx vs. barley Trx *h*) and *in vitro* reduction was performed at different temperatures (37 °C vs. 22 °C). In a further modification, Marchand et al. [74] used ^{14}C-labeled iodoacetamide (IAM) to visualize proteins reduced by Trx in extracts from *Arabidopsis* leaves. Of the about 40 Trx-

linked proteins identified, 21 were known targets at the time and 19 were newly identified. Proteins functioned in 11 cellular processes, including defense against pathogens and herbivores (4 proteins) and oxidative stress (5 proteins). Proteins also had different cellular origins, including cytosol, chloroplasts, mitochondria, vacuoles and apoplasm.

In subsequent independent studies, Maeda and Marchand and their collaborators identified the Cys residues reduced by Trx in certain targets by using differential labeling of reduced vs. oxidized Cys residues (see Section 6). More recently, a strategy based on the ICAT method was employed to identify all disulfides targeted by Trx in soluble proteins of barley seed
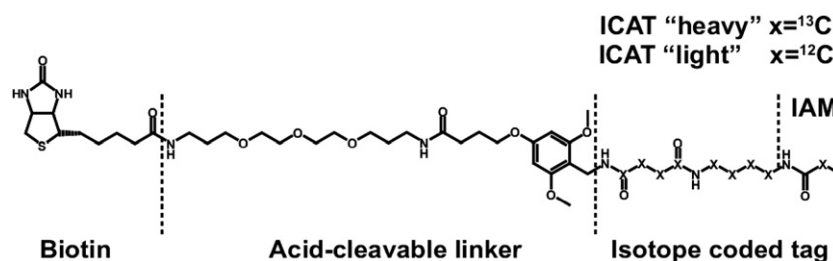
Fig. 3 – Structure of light and heavy tags used in the adapted ICAT strategy.

embryo [75]. This strategy provided differential labeling of thiol groups by using affinity tags that have either light, ^{12}C carbon or heavy, ^{13}C carbon (Fig. 3). The tag consists of IAM linked to biotin by an acid-cleavable linker. Briefly, thiol groups of proteins are blocked with IAM after incubation with NADPH and either NTR alone or NTR plus Trx. Remaining disulfides are chemically reduced by Tris(2-carboxyethyl) phosphine (TCEP), a thiol reducing agent, and the newly formed thiol groups are further derivatized by either the light tag (proteins treated with NTR plus Trx) or the heavy tag (proteins treated with NTR alone). Finally, the two samples are mixed in a 1:1 ratio and digested with trypsin. The resulting peptides are purified by affinity chromatography on an avidin column and, after removal of the biotin-linker moiety, analysed by LC/MS.

Because non-target disulfide bonds remain intact until TCEP addition, they give rise to identical amounts of thiol groups available for labeling in both samples. Thus, for peptides containing non-targeted Cys residues, the H/L ratio of peptides labeled with heavy tag to counterparts labeled with light tag is expected to be one. By contrast, when Cys residues are released after Trx treatment, they are blocked with IAM and hence not labeled with the light tag. The H/L ratio of peptides containing those residues was expected to be higher than 1. In fact, control experiments confirmed that an H/L ratio equal to or higher than a threshold of 1.22 is indicative of Trx reduction. Significantly, the ICAT adapted strategy allows not only the identification of the disulfide bonds in proteins targeted by Trx, but also a quantitative assessment of the efficiency of their reduction.

Using this approach, Hägglund et al. [75] identified 199 peptides labeled with the affinity tags, 106 of which correspond to 90 proteins linked to Trx. In addition, among the 40 disulfide proteins that were efficiently reduced by Trx—i.e., containing peptides labeled with an H/L ratio >1.5—several had been previously identified as Trx targets. An impressive number of ribosomal proteins were notably present among the 90 targets along with several proteins functional in amino acid synthesis, protein synthesis and folding. The work confirms and extends the key role that Trx plays in protein synthesis.

Most of the ribosomal proteins identified have a basic pI. Their absence in previous surveys based on 2D-gels is believed to be due to poor separation in IEF [75]. Enzymes functional in metabolism and stress response were also present—e.g., dehydroascorbate reductase, a participant in the ascorbate–glutathione cycle detected in previous proteomic studies. Interestingly, the catalytic Cys residue of this enzyme of barley embryo was the most extensively reduced target detected, underscoring the importance of Trx h in this cycle.

4.2. Targets isolated by mutant Trx affinity trapping

In vitro studies demonstrated early on that Trx, with a CxxC active site, reduces protein disulfide bridges in a two-step reaction [76]. In the first step, the more N-terminal catalytic Cys of Trx attacks the disulfide bridge of the target protein, reducing one Cys of the target and establishing a hetero-disulfide bridge with another. In the second step, the more C-terminal Cys of Trx attacks the bridge of the heterodisulfide intermediate, allowing the release of both the reduced target and the oxidized Trx. When the resolving Cys was replaced by Ser or Ala in mutant Trx proteins, the normally transient heterodisulfide was stabilized by a covalent link between the target protein and Trx, a bond that can be cleaved by DTT [77,78]. Such a mutant Trx was subsequently used to demonstrate the interaction between Trx and PRK and FBPase [79,80].

In the pioneering work of Verdoucq et al. [81], a yeast line that expressed a C35S (E. coli) mutant Trx was used to isolate stable heterodisulfide intermediates formed in vivo. They purified a complex comprised of the mutant Trx and a protein that had all the characteristics of Prx as well as Trx-dependent peroxidase activity. Motohashi et al. [82] built on this finding and devised a mutant Trx approach that is currently widely used to capture targets (Table 1). These investigators incubated a spinach chloroplast stromal lysate with mutant Trx m immobilized on a resin using the batch method. The Trx-resin was extensively washed, first with a high concentration of NaCl to remove non-specifically bound proteins and then with DTT to reduce the heterodisulfide bond and release captured target proteins (Fig. 4). Following SDS-PAGE, the eluted proteins were transferred onto a polyvinylidene diflouride membrane and their N-terminal sequence determined by Edman degradation. Among the nine proteins identified, six were established Trx targets in plants (NADP-GAPDH, 2-Cys Prx, Prx Q, RubisCO activase and SBPase) or algae (glutamine synthetase; [83,84]) and two were previously unknown targets (RubisCO small subunit and a cyclophilin, PPIase). The mutant form of Trx f was found to be considerably less effective in capturing potential targets than the Trx m counterpart.

Balmer et al. [85] applied the same approach to capture potential targets from stroma of spinach chloroplasts. This time, however, the trapped proteins eluted with DTT were separated by 2DE before their identification by mass spectrometry. Their approach led to the identification of 37 proteins, 15 functional in 10 chloroplast processes at the time not known to be Trx-linked, including the synthesis of isoprenoids, tetrapyrole and thiamine, replication of DNA and
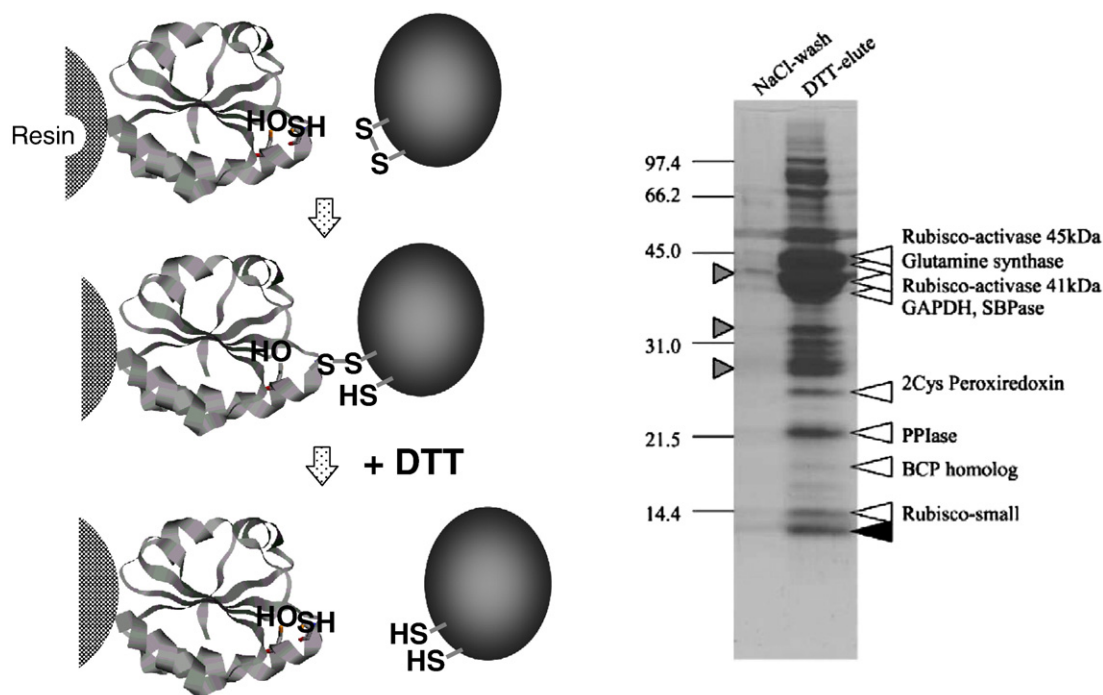
Fig. 4 – Isolation of Trx target proteins by mutant Trx affinity trapping. Left: Explanation of the process; Right: application to stromal proteins of spinach chloroplasts. Mutant Trx *m* with resolving Cys residue of the active site substituted with Ser was immobilized on the resin and used to trap Trx targets in the chloroplast stromal extract by the formation of stable mixed-disulfide bonds. The trapped target proteins were eluted with DTT, separated by SDS-PAGE and transferred on a membrane. Each of the protein bands was identified by N-terminal sequencing. White and gray triangles indicate proteins for which an N-terminal sequence was determined and either assigned (white) or not assigned (gray) in the database. The black triangle indicates the mutant Trx *m* that was released from the resin. The mass of the molecular markers is indicated on the left. From [82]. Copyright (2001) National Academy of Sciences, U.S.A.

division of plastids. Almost without exception, the Trx-linked proteins identified contained conserved Cys residues. A comparison of the targets trapped on the mutant Trx *f* and *m* columns revealed that, under these conditions, Trx lost the specificity that had been established in enzyme assays.

In a separate study, Yamazaki et al. [86] used mutant Trx *h* affinity columns to capture targets in an extract from etiolated *Arabidopsis* seedlings. Among the 15 proteins identified by 2DE and mass spectrometry, four were either previously known or proposed as targets in chloroplasts [82,85], indicating their presence in etioplasts. Two, NAD-dependent GAPDH and MDH, had been identified previously in a soluble protein fraction from wheat endosperm [72]. Nine new targets functioned in protein synthesis or turn over, cell metabolism, and stress defense.

As Trx *f* had long been known to form a noncovalent complex with FBPase [10], Balmer et al. [87] used wild-type Trx *f* affinity chromatography to determine whether other chloroplast proteins form ionic complexes. Twenty eight proteins were identified, among them FBPase, thereby providing evidence that Trx *f* can interact noncovalently with a spectrum of proteins. The complexes formed between members of the FTS and interacting proteins appear to insure efficient electron transfer from Photosystem I to the final acceptor metabolite or protein [87]. The finding that wild-type Trx *f* affinity chromatography failed to capture all of the known chloroplast targets indicates that some fail to form a stable electrostatic complex. The identification of 10 new targets, most known to belong to chloroplast protein complexes in which at least one component is linked to Trx, suggests that they interact indirectly with one of the target enzymes rather than directly with Trx *f* itself.

During this period, affinity chromatography with mutant Trx *m* or *h* was used to isolate soluble Trx-linked proteins of mitochondria from photosynthetic (pea and spinach leaves) and heterotrophic (potato tubers) sources [88]. Potential targets, that were similar irrespective of the type of Trx used, were separated by 2DE and 50 potential Trx-linked proteins were identified by mass spectrometry. Almost all proteins isolated could be reduced by Trx and detected by fluorescent gel electrophoresis using the method of Yano et al. [69]. Novel targets included proteins functional in photorespiration, citric acid cycle, lipid metabolism, electron transport, membrane transport and hormone synthesis.

Potential Trx targets have also been identified in cyanobacteria (*Synechocystis* PCC 6803) and green algae (*C. reinhardtii*) using affinity trapping (Tables 1 and 2). Although unicellular and of relatively early evolutionary lineage, *C. reinhardtii* has a large number of Trx targets in common with land plants. By contrast, *Synechocystis* shows differences. Thus, about 70% and 30% of the targets identified in green algae and cyanobacteria—i.e. 58 and 81 proteins or subunits, respectively—have orthologs in land plants. Five proteins are common to the three types of organisms:

glutamine synthetase, chaperonin 60 kDa, elongation factor Tu, Prx and RubisCO [89–94]. The apparent low percentage of potential targets shared by cyanobacteria and higher plants may be largely due to the high proportion of membrane proteins identified in cyanobacteria (see below) as well as the evolutionary distance between the organisms. It can also reflect differences in protein function and natural habitat [94].

Proteins potentially targeted by CDSP32 and HCF164, two Trx-like disulfide proteins having a functional Trx domain, were also trapped using mutant affinity columns (Tables 1 and 2). The six proteins trapped with mutant CDSP32 [95,96]—a protein induced during drought stress in chloroplasts of potato leaves [97]—were previously found to be linked to classical Trx: ATP synthase, RubisCO, two Prxs (Q and BAS1), and a B-type methionine sulfoxide reductase. Similarly, except for cytochrome f, all proteins trapped with the mutant HCF164 [98]—a protein functional in the assembly of cytochrome b_6f in thylakoid membranes [99]—are also targets in either land plants (ATP synthase, chlorophyll a/b-binding protein, Rieske, photosystem I reaction center subunit N) or cyanobacteria (FTSH).

4.3.   Targets identified using combined labeling and isolation strategies

Fluorescence gel electrophoresis and mutant Trx affinity trapping approaches have been applied jointly in several studies. In each case, the two approaches were found to be complementary. In an analysis of developing wheat seeds, Wong et al. [100] found that of the 68 targets identified, one-third were unique to either fluorescence labeling or mutant Trx affinity column chromatography, whereas one-third were detected by both procedures. Almost all proteins contained conserved Cys, and 40 had not been previously identified as potential Trx targets. The proteins functioned in cell division, amino acid synthesis and protein synthesis—processes characteristic of seed development [101]. As these and other complementary detection methods are refined, we will likely continue to uncover previously unexplored parts of plant proteomes.

In their analysis of the complete proteome of amyloplasts isolated from wheat seeds, Balmer et al. [102] found Fdx, Fdx-NADP reductase (FNR) and FTR among the 284 proteins isolated. This observation, together with the earlier demonstration that ADP-glucose pyrophosphorylase of potato tubers is regulated by Trx [47,103] prompted Balmer et al. [104] to search for other Trx-linked enzymes in isolated wheat amyloplasts. In this case, mBBr fluorescence labeling was used in combination with Trx affinity chromatography. They found that, like chloroplasts, amyloplasts, contain a complete FTS consisting of Fdx, FTR and an m-type Trx. In amyloplasts, Fdx is reduced not by light, but by metabolically generated NADPH via FNR. Thus, in the amyloplast redox system, electrons flow from NADPH to Trx and Target Proteins according the pathway identified for cyanobacterial heterocysts: NADPH→FNR→Fdx→FTR→Trx m→Target Protein. As found for developing seeds [100], approximately one-third of the proteins were detected individually by either fluorescence labeling or Trx affinity chromatography and one-third were recognized by both procedures. Of the 42 putative targets identified in amyloplasts [104], thirteen were previously unrecognized targets functional in protein assembly/folding as

well as in biosynthesis of starch, lipids, nucleotides, and amino acids.

Potential Trx targets were re-examined recently in leaves of *Arabidopsis* by Marchand et al. [105]. Here, targets were either isolated by affinity chromatography or labeled with two different thiol specific reagents (^{14}C-IAM or PEO-iodoacetylbiotin). Proteins labeled with biotin were further trapped on an avidin resin. Altogether the three approaches revealed 71 different proteins as being Trx-linked. This number is much higher than in the former study by this same group (44). Affinity chromatography and ^{14}C-IAM-labeling were both effective, respectively yielding 47 and 41 potential targets, and, as expected, complementary results. By contrast, PEO-iodoacetylbiotin labeling was much less successful with only 12 proteins recovered. Only three were common to the three procedures (myrosinase, PRK and NAD-MDH). At the time of the experiments, two new potential targets that function in tyrosine and cell wall synthesis were identified.

Finally, Alkhalfioui et al. [106] applied fluorescence labeling and affinity chromatography to identify potential Trx targets in seeds of *M. truncatula*. They identified 111 proteins as potential targets among which 30 were recovered by both procedures, 59 were new, 34 found previously in cereal or peanut seeds, and 18 in other plants or photosynthetic organisms. New processes possibly linked to Trx included the synthesis of folate and phenylpropanoids, the polymerization of DNA, the binding of ions or metabolites, and resistance to aluminium and desiccation.

In contrast to the above studies leading to the identification of a large number of potential Trx targets, the screen employed by Juarez-Diaz et al. [23] with *Nicotiana alata* style extracts yielded only a single candidate—a unique S-RNase—shown to be both reduced and able to interact with the native form of a particular Trx h that was found to be secreted in the extracellular matrix.

4.4.   Membrane proteins targeted by Trx

Owing to inherent technical difficulties in working with hydrophobic proteins, proteins linked to Trx has been mainly limited to soluble representatives. In a few studies, however, labeling or isolation approaches were adapted to identify Trx-linked proteins in membrane fractions. In one study, an adaptation of the approach based on the fluorescent thiol probe mBBr was applied to identify redox-linked proteins of thylakoid membrane of spinach chloroplasts [107]. Fourteen different proteins were identified, of which nine were integral membrane components including seven new candidates functional in processes associated with photosynthetic electron flow, ATP synthesis, and Photosystem II/Photosystem I state transitions.

In a more recent study, Bartsch et al. [108], extracted proteins from the inner envelope membrane of barley chloroplasts in the presence of 3% SDS, loaded them on Trx affinity columns (mutant Trxs f and m) and, after elution, applied proteomic analyses. Five major protein bands were observed in SDS-PAGE, and one of the more abundant, a 52 kDa component, was found to contain three novel targets: protein translocon TIC55; precursor of NADPH:protochlorophyllide oxidoreductase translocon protein PTC52, which operates as protochlorophyllide a

oxygenase; and pheophorbide a oxygenase (PAO), originally called the lethal leaf spot protein LLS1. TIC55 and PTC52 have established roles in protein translocation across the inner plastid envelope membrane while PAO is involved in chlorophyll metabolism. All three proteins, which contain redox-active CxxC motifs, interacted with mutant Trxs *f* and *m*. The ability of native Trxs to reduce the targets was confirmed with mBBr. Reduction by Trx *m* (or DTT) induced a 12- and 15-fold increase in PTC52 and PAO activities, respectively. Taken together, the findings suggest a role for Trx in the diurnal regulation of protein translocation and chlorophyll (Chl) metabolism. In this study, proteins from the outer envelope membrane and stroma were also subjected to affinity chromatography. Interestingly, a protein of unknown function related to Erv1 and Erv2, enzymes implicated in disulfide bridge formation in mitochondria and bacteria, was isolated from the outer envelope membrane. Among the proteins isolated from the stroma, a few were verified as being Trx targets.

The finding that only a few proteins extracted from the membranes interacted with mutant Trxs under the conditions used is likely due to the denaturing effect of SDS. On the other hand, the solubilization of membrane proteins prior to contact with a mutant Trx may expose buried disulfides not normally accessible to Trx. To overcome this problem, Mata-Cabana et al. [109] developed a procedure in which a His-tagged mutant Trx was added to an intact membrane extract to allow the binding of potential targets to mutant Trx *in situ*. Following membrane solubilization, mutant Trx complexes were isolated by Ni-affinity chromatography and resolved in 2D-diagonal gels (non-reducing/reducing SDS 2DE). By applying this method to total (thylakoid and plasma) membranes from the cyanobacterium, *Synechocystis*, 50 Trx-interacting proteins were isolated. Among the 38 newly identified targets, were the ATP-binding subunits of several transporters and members of the AAA-family of ATPases.

In an earlier study, Lee et al. [110] analyzed trichloroacetic acid (TCA) precipitates of *Arabidopsis* stem and leaf extracts in an attempt to identify all disulfide proteins potentially regulated by Trx. An advantage of TCA is that it precipitates both soluble and membrane proteins in their native redox state. Sequential alkylation of free thiols, chemical reduction of disulfides by DTT and isolation of proteins by thiol affinity chromatography were then performed in the presence of SDS. Of the 65 disulfide proteins identified by mass spectrometry, 20 were unknown potential redox-active proteins. Among the 27 chloroplast proteins identified, most were either confirmed or previously proposed Trx targets. In addition to nine of the cytoplasm, 29 were targets predicted to be processed via a secretory pathway.

In yet another approach, Winger et al. [4] isolated mitochondria from cultured *Arabidopsis* cells and used 2D-diagonal gels to identify proteins able to undergo thiol-disulfide exchange *in vitro*. SDS was present in both dimensions along with diamide as oxidant in the first dimension and DTT as reductant in the second. Of the 18 proteins identified, 12 were previously recognized Trx targets. Use of SDS-solubilization resulted in the identification of integral membrane proteins. Indeed, four of the six newly identified targets were membrane or membrane-associated proteins, including two that have six transmembrane domains. Similarly, in a recent study Ströher and Dietz

[111] analyzed the thiol-disulfide redox proteome of thylakoid-bound, luminal and stromal fractions of chloroplasts from *Arabidopsis* using protein extracts treated with DTT or H_2O_2. The 49 polypeptides identified included 22 candidate targets that have not been recognized previously. Among these proteins were PsbA, PsaA1 and PsaF, chloroplast monodehydroascorbate reductase as well as Deg1 protease. Monodehydroascorbate reductase was soon thereafter also recovered in the screen of Hägglund et al. [75] as mentioned above. Recombinant Deg1 and Deg2 revealed redox dependence of their proteolytic activity. A possible electron donor for Deg1 protease might be HCF164.

The methods described above were applied to identify all proteins containing a redox-active disulfide. However, because they were not shown to interact with Trx, these proteins were not included in the list of targets presented in Table 2. It remains to be seen whether some may be either actual Trx targets or, alternatively, be modified by glutathionylation or nitrosylation and targeted by Grx.

5. Advantages and limits of proteomic approaches

Separation of proteins by 2DE following thiol labeling or affinity chromatography is a powerful method for resolving potential Trx targets. Comparison of 2D-gel patterns in control gels with patterns observed when proteins are reduced by a thiol-specific probe enables the detection of authentic redox-regulated targets that can then be identified by Edman microsequencing or mass spectrometry. One drawback lies in the fact that spots in 2D-gels may contain more than one protein. Thus, while unequivocal identification of all target(s) is not possible with current methodology, the spots reveal previously unknown candidates that can be verified in future studies. Due to the range of protein abundance in eukaryotic cells and the limited amount of protein that can be resolved by 2DE, only the most abundant targets can presently be identified in cell extracts by proteomic approaches. Less abundant counterparts can often be identified when extracts are partially purified or organelle fractions are isolated prior to thiol labeling or affinity chromatography. Enrichment also reduces the problem of recovering multiple proteins in single gel spots.

The ICAT technique, which is based on the analysis of tagged peptides, should theoretically lead to the identification of all potential Trx targets provided the tryptic peptides generated can be detected by LC/MS. This technique permits the identification of proteins of low abundance or with extreme pI such as ribosomal and membrane proteins that are poorly separated on 2D-gels. Perhaps more important, the technology uniquely enables the identification of Cys residues targeted by Trx as well as the efficiency of their reduction.

The labeling approaches have the potential advantage to be adapted to monitor the *in vivo* redox state of proteins and elucidate redox changes taking place during development or under fluctuating environments [106,112]. Notably, they can also yield quantitative results. Indeed, a thiol-labeling technique based on ^{14}C-NEM combined with two-dimensional gel

analysis was employed with success by Leichert and Jakob in 2004 [113] and Le Moan et al. in 2006 [114] to assess the redox state of proteins in *E. coli* and yeast, respectively. Proteins undergoing thiol modification during oxidative stress were also identified. With this technique, ^{14}C-NEM was used to label Cys newly formed upon DTT reduction either before (labeling of total accessible Cys of a given protein) or after treatment with IAM (labeling Cys that were oxidized *in vivo*). Then, the ratio of oxidized vs. total accessible Cys was calculated for each radioactive protein detected in the gels, also taking into account the total number of Cys residues per molecule. Le Moan et al. [115] recently compared results obtained with radioactive NEM with those obtained with fluorescent NEM in 2D differential gel electrophoresis (2D-DIGE) and found that the latter method was accompanied by a relatively high noise background. Also recently, Leichert et al. developed a gel free quantitative method based on ICAT strategy named oxICAT [116]. Prior to trypsin digestion, the free SH groups present *in vivo* were labeled with the light (^{12}C) tag. Next, proteins were treated with TCEP and the SH groups newly formed were labeled with the heavy (^{13}C) tag. Peptides generated by trypsin digestion were then analysed. To our knowledge, no corresponding quantitative values are available for photosynthetic organisms. However, the method designed by Hägglund et al. may be readily adapted for this purpose by reducing the proteins with TCEP instead of NTS.

Experimental conditions used to label or trap targets may possibly lead to the identification of proteins that are not redox regulated. In some tissues, proteins may be partially or highly reduced *in vivo* and, consequently, must be oxidized prior to labeling or affinity isolation using, e.g., H_2O_2 or extended exposure to air [70,106]. Thus, in addition to authentic targets, the experimental conditions could result in false positives. Further, the concentration of Trx used in labeling or isolation is much higher than the endogenous levels existing in plant cells. In this way, reactions that are untenable *in vivo*, could occur *in vitro*. Thus, proteins with exposed free Cys that possibly are not redox regulated could be reduced by native Trxs or trapped on mutant Trxs. Finally, mutant Trxs may also react with glutathionylated or non-specific oxidized Cys residues [117,118].

Trx specificity continues to be an issue. This aspect was highlighted in early heterologous complementation experiments with *Arabidopsis* Trx h and m isoforms using the Trx-deficient yeast strain mentioned above [119,120]. Specificity was further confirmed following the two-hybrid approach with a yeast strain recently designed by Vignols et al. [121]. However, as noted above, proteomic procedures applied to screen for Trx targets are generally characterized by lack of Trx isoform specificity, and yield potential targets irrespective of the type of Trx used (*m, f, h, E. coli*). Some Trx-linked proteins were even trapped by mutant forms of Trx-like CDSP32 and HCF164, which themselves are Trx targets [95,98]. An exception to this rule appears to be the screen for targets in *N. alata* style extracts [23] where S-RNase was the sole protein interacting with the particular Trx h isoform secreted in the extracellular matrix.

Specificity for Trx vs. Grx can also be challenging. Rouhier et al. [118] used mutant Grx affinity chromatography to isolate 94 putative targets from poplar extracts. Included

were proteins found previously to interact with Trx. Similar results were obtained with *Synechocystis* proteins trapped with mutant Grx [122], suggesting that certain redox-active proteins may interact with more than one dithiol reductant. In addition, some of the proteins described as Trx or Grx targets were also found among the glutathionylated proteins recovered in *C. reinhardtii* [6]. A further complication stems from the fact that some well characterized plant enzymes, including PAPS reductase, Prxs and glutathione peroxidases, are able to use both Trx and Grx as electron donors. An overlapping of redoxin function can also be observed *in vivo*. Insertion mutants of *Arabidopsis* for NTRA or NTRB revealed that both genes encode mitochondrial and cytosolic isoforms of NTR [30]. However, the strains failed to produce a phenotype, indicating that these isoforms share redundant functions. Unexpectedly, plants of the ntra ntrb double knockout mutant were viable and fertile, demonstrating that neither mitochondrial nor cytosolic isoforms of NTR are essential [123]. Since a substantial fraction of the cytosolic Trx was found in the reduced state in the ntra ntrb mutant, it was suggested that GSH and Grx are involved in an alternate reduction of Trx.

6. Target validation approaches

To validate their role as authentic targets, candidate Trx-linked proteins identified by thiol labeling and affinity chromatography must be verified biochemically or genetically. The inclusion of previously established targets in experiments designed to identify unknown candidates constitutes encouraging evidence that the candidates are redox regulated. For example, Balmer et al. [85] used mutant Trx *f* and *m* affinity chromatography to trap nine well known and 11 previously unknown targets in chloroplasts. However, seven other previously identified targets were not retained on the column, among them FBPase. While the reason for the absence of six of the known targets remains a mystery, the failure to detect the FBPase was thought to be due to the extra (third) Cys present in the redox active site of the protein. While bound to the mutant Trx, this residue likely formed a disulfide with one of the Cys participating in the heterodisulfide bonding with Trx, thereby displacing the FBPase enzyme from the column [85].

The presence of Cys residue(s) that are conserved among the orthologs in related species provides preliminary evidence for putative targets. One or ideally two conserved Cys are required because Trx rather acts on intra- than on inter-molecular disulfides. When only one conserved Cys residue is present in the primary structure of a candidate, it is possible that the protein is a subunit of a dimer linked by a disulfide that is targeted by Trx as is the case for ADP-glucose pyrophosphorylase [47]. Another well known target, 2-Cys Prx in yeast, also exists as a dimer of identical 25 kDa subunits, each having only one conserved Cys [124]. In a few cases, proteins without conserved Cys residues are identified. One explanation for this observation is that affinity chromatography can possibly trap these proteins through an electrostatic interaction with an authentic Trx target or with Trx itself. Similarly, putative targets lacking Cys can be identified in a

labeled gel spot that contains co-migrating proteins if sequence information for genuine Trx target(s) is lacking in that spot.

To confirm reduction by Trx, the putative target should ideally be purified from the native source or produced as a recombinant protein and assayed biochemically. Trx should alter properties of the target and be a much more effective reductant than either GSH or reduced Grx. Early on, Kobrehel et al. [52] used mBBr to show that Trx was more effective than GSH in reducing proteins from wheat seeds. More recent biochemical assays with candidate targets identified by proteomic approaches from wheat flour preparations revealed that Trx, reduced by DTT, effected a 1.3- to 12.7-fold increase in enzyme activity of targets that included alanine amino-transferase, aldolase, enolase, NAD-GAPDH, pyruvate:phosphate dikinase and triose phosphate isomerase [72]. When tested for redox-linked changes in separate studies, the activity of recombinant preparations of alcohol dehydrogenase, NAD-GAPDH, NAD-MDH [125] as well as Prx Q and the cyclophilin ROC4 [126] was altered by Trx.

However, in one case, the biochemical assay of the recombinant protein was negative. Using recombinant ribose-5-phosphate isomerase, a target identified in both *Arabidopsis* chloroplast and *C. reindhardtii* screens (Table 1), Hisabori and co-workers [127] were unable to demonstrate reduction by Trx. Further work is needed to explain the presence of such a protein in the two screens and determine whether Trx interacts and alters the protein in a way yet-to-be identified. Regulation of activity by glutathionylation is another possibility.

When available, structural features around disulfide bonds in potential targets may help to discriminate Trx targets from those of GSH, Grx or GST [128].

Demonstrating change in the redox status of potential target proteins *in vivo* provides an alternate method for validation. Apropos this point, Yano and Kuroda [112] found that a soluble extract from rice bran (enriched in aleurone layer) reduced endogenous Trx and target proteins in the presence of NADPH and NTR when change was monitored with mBBr (Fig. 5). Under these conditions, the isoforms of ESP, one of the potential targets of bran visible in the control Coomassie stained gel (Fig. 5, Bottom, spots 1, 2, 3), disappeared when reduced by Trx. Globulins (spots 4, 5), other major Trx target proteins, by contrast, remained even when reduced as evidenced by the highly fluorescent spots (Fig. 5, Top). When reduction was conducted in the presence of leupeptin, a Cys-protease inhibitor, ESP remained even in reduced form (Fig. 5). GSH showed no effect. ESP was also reduced during germination. The authors concluded that ESP is Trx-linked. Further, when reduction took place *in vivo*, ESP was degraded by an unidentified Cys-protease that was also activated by Trx. The results exemplify once again that reduction of Trx targets can lead to an increase in protease susceptibility (ESP) or to a change in activity (Cys-protease). Similarly, in a screen with *M. truncatula* seeds, Alkhalfioui et al. [106] demonstrated that candidate (and established) Trx targets of legume seeds, which were reduced specifically by Trx *in vitro*, were also reduced *in vivo* during germination.

Demonstrating that an interaction between a potential target and Trx can take place *in vivo* provides alternate validation of a candidate as a true target. To this end, Vignols et al. [121] used the two-hybrid approach with the yeast strain they designed to confirm known targets. Significantly, this strain can also be used to discriminate different Trx isoforms.

Once a protein is shown to be a true Trx target, the next step is to identify the Cys residues functional in redox regulation. These Cys residues have been identified for a growing number
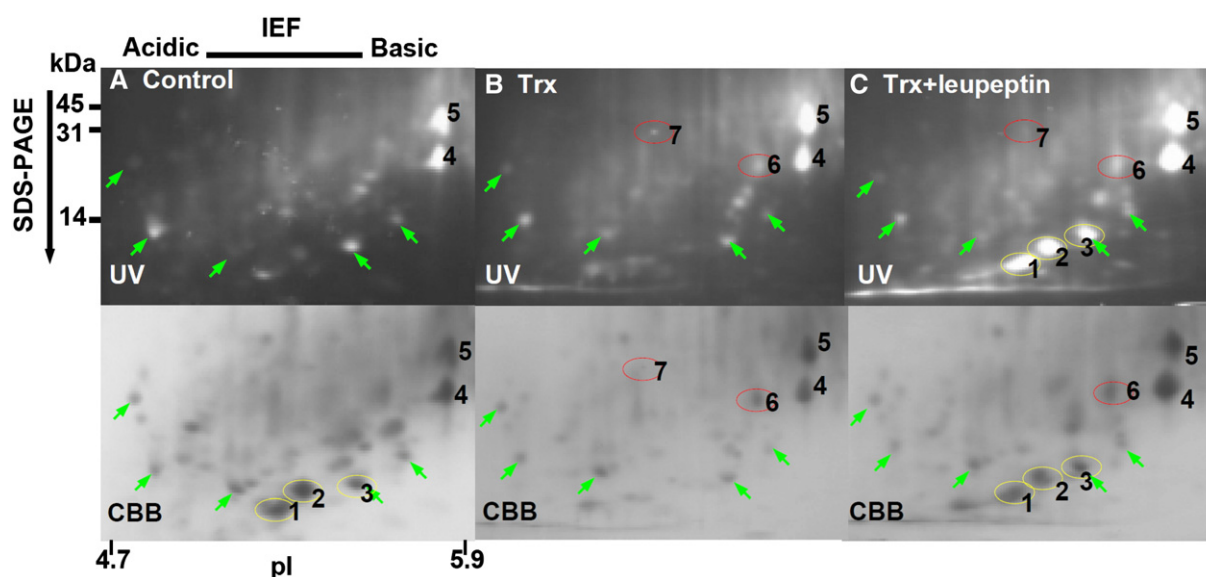

Fig. 5 Reduction of embryo-specific protein by Trx followed by degradation by a non-identified, Trx-dependent Cys-protease. Buffer soluble rice bran proteins were incubated under the following conditions. (A) Control, no addition with 3 h incubation. (B) Trx, incubation for 3 h in the presence of NADPH and *E. coli* NTR. (C) Trx+leupeptin, incubation under the same conditions as B, except in the presence of leupeptin. After separation by IEF/SDS 2DE, fluorescent proteins were examined under a UV lamp (UV: top dark panels) before the staining of proteins with Coomassie brilliant blue (CBB: bottom grey panels). 1, 2, 3: Embryo-specific protein; 4, 5: Globulins. Green arrows represent proteins used as markers. From [112]. Copyright (2006) Proteomics.

of individual targets uncovered by proteomic approaches. Motohashi et al. [126] used protease digestion, HPLC separation, N-terminal peptide sequencing, and mass spectrometry to identify the Cys residues involved in redox regulation of the cyclophilin ROC4. They confirmed the formation of disulfide bonds by quantifying the number of free sulfhydryl groups in the oxidized and reduced forms of the protein with DTNB (5, 5′-dithio-bis(2-nitrobenzoic acid). Comprehensive studies have also been carried out by using differential labeling of reduced vs. oxidized Cys residues. Maeda et al. [129] applied this method to detect Trx *h*-reducible disulfides in proteins of barley seeds. Proteins were treated with IAM to block the accessible thiol groups before reduction by Trx *h*. Newly formed thiol groups were derivatized with 4-vinylpyridine, and proteins were separated by 2DE and analyzed by LC/MS to distinguish thiols from disulfides. Mass shifts of 15 peptides revealed 9 redox-active disulfides in the α-amylase/subtilisin inhibitors. Similarly, Marchand et al. [105] used IAM/PEO-iodoacetylbiotin or IAM/4-vinylpyridine in differential Cys labeling to identify the redox active disulfides in 13 of their reported Trx targets. Using the ICAT strategy, Hägglund et al. [75] were able to determine the redox active disulfide of most of the potential targets identified.

Finally, as typically found with chloroplast targets identified in biochemical studies during the first 25 years following the discovery of the FTS before the advent of proteomics, mutagenesis of the active Cys residues usually alters the activity of target enzymes. Using mutagenesis, Motohashi et al. [126] further confirmed the importance of the active Cys residues in ROC4 to the peptidyl-prolyl cis-trans isomerase activity of the enzyme. However, as noted above, so far relatively few targets identified by proteomic approaches have been validated up to this point. This gap remains a challenge for the future. As previously unrecognized targets are confirmed, it will be of interest to determine how they interact with Trx. The structural picture that emerged from the work of Maeda et al. [128] on the complex formed between barley Trx *h* and α-amylase subtilisin inhibitor target will serve a guide for future work in this area.

To sum up, validation of proteins identified as Trx targets relies on establishing the presence of conserved Cys in their primary structure; the effect of reduction on activity or other properties (e.g., protease sensitivity, solubility, heat stability); specificity for Trx vs. GSH/Grx; identification of the Cys residues active in redox modulation; and, finally, the effect of mutagenesis of Cys residues on protein function.

7.   Concluding remarks

For the first 25 years following the identification of the chloroplast ferredoxin/thioredoxin system, a relatively small number of proteins were found to be linked to Trx (<40 in plants). The advent of proteomics changed the landscape dramatically. Currently, approximately 500 proteins have been identified as potential or established Trx targets in oxygenic photosynthetic organisms (land plants and oxygen-evolving microorganisms) (Table 2). While important unanswered questions remain, it seems certain that regulation by Trx and redox embraces virtually all life processes. Our

understanding of these processes will be progressively enriched as quantitative methods are applied to organisms living under stable conditions as well as to counterparts undergoing development and adaptation to environmental change.

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